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Review

Immunotherapy of Type 1 Diabetes: Where Are We and Where Should We Be Going?

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Type 1 diabetes (T1D) is a chronic autoimmune disorder characterized by destruction of insulin-producing pancreatic β cells. Many broad-based immunosuppressive and antigen-specific immunoregulatory therapies have been and are currently being evaluated for their utility in the prevention and treatment of T1D. Looking forward, this review discusses the potential therapeutic use of antigen-specific tolerance strategies, including tolerance induced by "tolerogenic" antigen-presenting cells pulsed with diabetogenic antigens and transfer of induced or expanded regulatory T cells, which have demonstrated efficacy in nonobese diabetic (NOD) mice. Depending on the time of therapeutic intervention in the T1D disease process, antigen-specific immunoregulatory strategies may be employed as monotherapies, or in combination with short-term tolerance-promoting immunoregulatory drugs and/or drugs promoting differentiation of insulin-producing β cells from endogenous progenitors.

Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disorder thought to be caused by proinflammatory autoreactive T cells, which mediate the destruction of insulin-producing pancreatic β cells via both direct and indirect mechanisms leading to life-long dependence on exogenous insulin (Atkinson and Eisenbarth, 2001). Development of T1D is genetically controlled and thought to be initiated in susceptible individuals by environmental factors such as virus infections, although a viral cause has not been clearly identified (von Herrath, 2009). Although both humoral and cell-mediated immune mechanisms are active during diabetes, $CD4^+$ T cells occupy a critical role in T1D pathology (Anderson and Bluestone, 2005), as exemplified by the observation that the majority of the genes associated with elevated disease risk relate to the function of $CD4^+$ Th cells (e.g., a trio of major histocompatibility complex class II [MHC II] alleles [Concannon et al., 2009]). Prior to diagnosis of overt T1D, the pancreatic islets are infiltrated by inflammatory cells including $CD4^+$ T cells (Kent et al., 2005), and antibodies to various β cell antigens are demonstrable in the sera of patients at risk (Achenbach et al., 2005).

Because of the ocular, circulatory, cardiovascular, and neurological risks associated with hyperglycemia, treatments that prevent the pathologic autoimmunity from destroying pancreatic tissue is preferable to long-term management of symptoms by insulin replacement therapy because use of exogenous insulin cannot match the precision of endogenous insulin secretion. Much of what is understood about the pathogenesis and regulation of T1D has emerged from the study of spontaneous disease in the nonobese diabetic (NOD) mouse. NOD studies have highlighted the critical role of adaptive immune responses in disease pathogenesis as well as identifying various targets that prevent diabetogenic autoimmune responses as prime therapeutic candidates (Atkinson and Leiter, 1999; Shoda et al., 2005). However, it is critical to understand that there are numerous differences in the pathogenic mechanisms driving the initiation and progression of disease in the NOD mouse versus human type 1 diabetics, e.g., major differences in the antigens targeted and the composition of inflammatory cell infiltrates in the two species, as well as greatly increased expression of MHC class I in humans (Gianani et al., 2010).

Existing and emerging therapies aimed at regulating the autoimmune response largely involve broad-based immunoregulatory strategies, including the inhibition or deletion of lymphocyte subsets and/or the use of agents proposed to induce or re-establish immune tolerance via activation of regulatory T (Treg) cells, e.g., nonmitogenic anti-CD3 or antithymocyte globulin (Chatenoud, 2003; Chatenoud et al., 2001; Chung et al., 2007; Kohm et al., 2005). Some of these have shown efficacy in initial clinical trials, but there are risks with any of the broad approaches such as cytokine release and/or reactivation of latent viruses. A highly desired alternative approach is the attempted induction of antigen-specific tolerance to β cell antigens for prevention of disease development in patients at risk or in new-onset patients. This review will discuss immunoregulatory strategies employed as monotherapies or in combination, including the use of antigen-specific tolerance strategies, which are under evaluation in clinical trials and/or are being developed based on demonstrated efficacy in preventing or ameliorating disease progression in the NOD mice.

There are numerous pitfalls to the translation of laboratory findings to the clinic. Trials of therapies that alter the natural history of T1D have been hampered by the lack of biomarkers of the immune processes that cause the disease. There are immunologic "readouts" that correlate with the presence of T1D; for instance, the presence of autoantibodies against islet

cell antigens including glutamic acid decarboxylase 65 (GAD65), insulin, islet cell antigen 512 (ICA512), and more recently zinc transporter 8 (ZnT8) has supported the autoimmune nature of the disease and has clearly differentiated T1D from type 2 diabetes where these markers are not found (Seyfert-Margolis et al., 2006). More recently, cellular proliferation assays to islet-specific proteins have distinguished responses in patients from normal control subjects (Herold et al., 2009). Other assays have identified antigen-specific cells in the circulation (Pinkse et al., 2005). However, the direct causal relationship between these measures and disease has not yet been established. For instance, in studies in which glycemic control has been modified (e.g., Cyclosporin A [CSA] or anti-CD3 monoclonal antibody [mAb]), there were no identified changes in titers of autoantibodies (Bougnères et al., 1988; Herold et al., 2002, 2005; Keymeulen et al., 2005). Thus, an assay that would reflect tolerance to the immune process in T1D is not currently available but is highly sought after.

Immunologic assays may be used as measures of the effects of immune therapies, but their relationship to the disease process remains speculative. One is left with metabolic parameters as endpoints. Although the relationship of these endpoints to the clinical situation is clearer, it is important to recognize that the most widely employed studies are functional, not anatomic. For example, in murine studies of treatment with CD3 mAb at the diagnosis of T1D in NOD mice, improvement in insulin secretion reflected the recovery of degranulated β cells rather than growth of new cells (Sherry et al., 2006). Even the relationship between improved metabolic function and the sequelae of the disease is controversial, but clinical data have suggested a direct relationship between the two (Palmer et al., 2004).

Chemical- and Antibody-Mediated Therapies

Initial clinical studies for treatment of T1D involved small-molecule inhibitors with biologics undergoing evaluation in the past decades. These clinical trials have had successes and failures as summarized in Table 1. The following narrative explains the basis for and findings of these trials.

Cyclosporin A

CSA was employed in the first trials showing effects of immune therapies on T1D. Continuous CSA treatment initiated soon after diagnosis eliminated the need for exogenous insulin (Bougnères et al., 1988; Stiller et al., 1984). However, the lack of lasting effects and renal toxicity of the drug diminished enthusiasm for this approach and other broad-spectrum immune-modulating agents such as Azathioprine and Prednisone (Bougnères et al., 1990; Silverstein et al., 1988).

CD3 Monoclonal Antibody

CD3 mAb without Fc receptor (FcR) binding was developed with the goal of reducing T cell activation but maintaining immunoregulatory capacity in vivo via suboptimal TCR signals and/or induction of Treg cells. Preclinical studies indicated, however, that not only was in vivo activation quantitatively reduced, but the signal delivered by the modified Ab was also qualitatively different from FcR binding mAb (Belghith et al., 2003; Smith et al., 1997, 1998). These studies indicated a selective inhibitory effect on differentiated Th1 cells, which had been thought to be involved in β cell destruction. Rather than a direct inhibitory effect of the drug, which would require the continued presence of the agent, tolerance was achieved probably via induction of Treg cells. Disease did not recur over time after short-term treatment of newly hyperglycemic mice, and if treated mice did not completely reverse hyperglycemia after drug treatment, they did not destroy syngeneic transplants after anti-CD3 mAb treatment (Chatenoud et al., 1994, 1997).

FcR nonbinding anti-CD3 mAbs carrying mutations of the IgG1 Fc chain or with elimination of glycosylation sites [hOKT3-γ1(Ala-Ala) and aglycosyl anti-CD3] were found to be less activating than OKT3 (Bisikirska et al., 2005; Herold et al., 2003; Xu et al., 2000). In two trials, brief treatment of new-onset T1D patients was shown to attenuate loss of β cell function for ≥ 2 years (Herold et al., 2002, 2005; Keymeulen et al., 2005). Clinical parameters including hemoglobin A1c and insulin usage improved. Importantly, there was no evidence for long-term immune suppression. Circulating T cell numbers recovered to pretreatment levels by one month after treatment and the drug was well tolerated—the cytokine storm had largely been eliminated, although about 10% of subjects discontinued drug because of adverse events attributed to cytokine release. In the European trial, in which the number of circulating T cells was less than that in the North American trial, Epstein-Barr virus (EBV) reactivation was seen, but in all cases the infection resolved, and the reduced numbers of circulating lymphocytes were transient.

Children who are relatives of patients with T1D and have islet cell autoantibodies are at extraordinarily high risk for progression to diabetes. About 90% of subjects who meet these criteria, identified in the Diabetes Prevention Trial-1 (DPT-1), will have clinical disease within 7 years, and the median time to disease onset is 3.31 years (Sherr et al., 2008). The progression of β cell destruction in these individuals, therefore, resembles those with disease, and therefore because of the near certainty that disease will progress, interventions that have shown efficacy in subjects with diabetes could be considered in this group. Accordingly, TrialNet has initiated a trial of anti-CD3 mAb treatment in individuals at high risk of diabetes. Based on information from clinical trials in patients with the disease, the suggested outcome is maintenance of insulin secretion and prevention of disease onset.

The mechanism of drug action in patents is not resolved but may differ from that described in NOD mice. In this regard, Herold et al. (2003) isolated IL-10-producing $CD4^+$ cells from the circulation of drug-treated patients, and there was an increase in the relative ratio of production of IL-10:IFN-γ in patient cells activated ex vivo. An increase in adaptive $CD4^+CD25^+Foxp3^+$ cells that inhibit immune responses through a TGF-β-dependent mechanism has been found in the pancreatic draining lymph nodes of anti-CD3-treated mice, even in the absence of naturally occurring Treg cells (i.e., in $CD28^{-/-}$ mice) (Belghith et al., 2003; Bisikirska et al., 2005). It is not clear whether the same cells can be found in the circulation of patients. It has also been suggested that the mAb induces adaptive $CD8^+$ Treg cells whose mechanism of inhibition is not clear and may be similar to $CD8^+$ suppressor cells described in other clinical settings. The absence of a tolerance biomarker or even a functional assay that correlates with the pathogenic process has made it difficult to answer whether the drug induces tolerance in patients.

Table 1. Summary of Successful and Unsuccessful Immunotherapy-Based Approaches in Type 1 Diabetes and Relevant Animal Models

Therapy	Efficacy in Animal Model	Comments	Principle Adverse Events	References
Successful Clinical Trials				
Cyclosporine A	+ NOD mouse, BB/W rat	Continued use of the drug was needed	Renal toxicity	(Assan et al., 1994; Bougnères et al., 1988, 1990; Feutren et al., 1986; Laupacis et al., 1983; Mori et al., 1986; Stiller et al., 1984)
Antithymocyte globulin (alone or with Prednisone)	+ NOD mouse with Exendin-4, not as single agent	Also part of a hematopoietic stem cell transplant protocol	Thrombocytopenia, serum sickness	(Eisenbarth et al., 1985; Ogawa et al., 2004; Saudek et al., 2004; Simon et al., 2008; Voltarelli et al., 2007)
Anti-CD3 mAb	+ NOD mouse	Late timing was an issue in the first report but not in the second report	Mild transient cytokine release, transient EBV reactivation	(Chatenoud et al., 1994, 1997; Herold et al., 1992, 2002, 2005; Keymeulen et al., 2005)
Rituximab	+ NOD mouse		Grade 1 or 2 infusion-related reactions	(Hu et al., 2007; Pescovitz et al., 2009)
Etanercept	+ NOD mouse but depending on timing: − for older mice	Pilot human trial	No significant drug-related adverse events	(Jacob et al., 1990; Mastrandrea et al., 2009)
GAD65	+ NOD mouse	Only in those with diabetes < 6 months duration	Mild site irritation, no significant drug-related adverse events	(Agardh et al., 2005; Ludvigsson et al., 2008; Tian et al., 1996; Tisch et al., 1993)
Oral insulin (Prevention)	+ NOD mouse	Only a subset of prediabetic subjects with high IAA titer	No significant drug-related adverse events	(Skyler et al., 2005; Zhang et al., 1991)
Closed-loop insulin		A "biostator" (closed-loop system) was used and suppressed endogenous insulin production. No immune therapy was given	Hypoglycemia	(Shah et al., 1989)
Unsuccessful Clinical Trials				
Nicotinamide	+ NOD mouse		No significant drug-related adverse events	(Gale et al., 2004; Kolb and Burkart, 1999; O'Brien et al., 2000; Yamada et al., 1982)
Intranasal insulin	+ NOD mouse		Nasal irritation and discharge, cough, fever, GI symptoms	(Bonifacio et al., 2008; Every et al., 2006; Harrison et al., 2004; Näntö-Salonen et al., 2008)
Parenteral insulin	+ NOD mouse	A pilot clinical trial showed efficacy	Chemical hypoglycemia	(Atkinson et al., 1990; Diabetes Prevention Trial–Type 1 Diabetes Study Group, 2002)
Oral insulin	+ NOD mouse	See above. A change in the IAA titer for inclusion appeared to result in dilution of the drug effect	No significant drug-related adverse events	(Skyler et al., 2005; Zhang et al., 1991)
Insulin in incomplete Freund's adjuvant	+ NOD mouse	Small pilot trial, generated high titers of insulin antibodies	No significant drug-related adverse events	(Orban et al., 2009; Skyler et al., 2005; Zhang et al., 1991)

Antithymocyte Globulin

Antithymocyte Globulin (ATG) with prednisone had been shown to reduce insulin requirements in a pilot trial involving new-onset patients but was discontinued because of thrombocytopenia (Eisenbarth et al., 1985). In a more recent study, ATG (Fresinius) retarded the loss of C peptide (which correlates with loss of pancreatic β cell function) in new-onset patients without the need for continuous drug administration (Saudek et al., 2004). The importance of the multiple specificities of ATG compared to anti-CD3 or other anti-T cell mAbs is not known—CD3 is an important target of ATG, but ATG causes a more prolonged peripheral T cell depletion. Thus the effects of these two biologics on the T cell repertoire may be different.

Anti-CD20

Anti-CD20 (Rituximab) was recently employed in a T1D trial. B lymphocytes were first thought to be important in the initiation

of insulitis because the islets were clear of inflammatory lesions in B cell-deficient NOD mice (Serreze et al., 1996). Previous evidence, however, had questioned B cell-directed therapeutic approaches in established disease because it was possible to adoptively transfer disease with diabetogenic splenic T cells into NOD.SCID recipients, lacking B cells and antibodies (Miller et al., 1988). Hu et al. (2007) and Xiu et al. (2008) recently showed that diabetes was prevented in NOD mice by depleting B cells with CD20 mAb before and at the time of onset of hyperglycemia (9–12-week-old mice) and even reversed disease in about 30% of animals at the appearance of hyperglycemia. Interestingly, cotransfer of B cells from the successfully treated mice diminished the rate of adoptive transfer of disease, suggesting a possible role for activation of "regulatory" B cells. Others have since shown that IL-10-producing B cells can be induced in mice depleted of $CD20^+$ B cells (Yanaba et al., 2008).

A recent randomized placebo-controlled trial of CD20 mAb (Rituximab) showed modest (23%) but significant improvement in β cell function 3 months after diagnosis and overall at 1 year, in drug-treated compared to placebo-treated subjects (Pescovitz et al., 2009). There were also significant improvements in clinical parameters including hemoglobin A1c and insulin use. After 3 months, however, there was a parallel decline in β cell function in the drug- and placebo-treated subjects. Subtle but significant differences in the depletion of $CD19^+CD27^+IgD^+$ cells differentiated clinical responders from nonresponders. However, there was little evidence that the drug induced immunologic tolerance. The $CD19^+$ cells, which reached a nadir level at study month 1, had not recovered to control levels after 12 months, and the levels of IgM were still significantly depressed. Once again, maintenance of clinical efficacy will require either a combination of drugs or repeated treatment, but the chronicity of immune suppression is of concern.

Cytokine- and Cytokine-Receptor-Directed Therapies

Cytokine- and cytokine-receptor-directed therapies are also in development for treatment of T1D. Human insulitis shows a considerably greater infiltration of innate immune cells such as macrophages and natural killer (NK) T cells compared to NOD insulitis (Dotta et al., 2007; Itoh et al., 1993). Moreover, innate mediators (TNF-α, IL-1, and type 1 interferons) were among the first molecules shown to have direct cytotoxic effects on β cells and were postulated to be the direct cause of β cell killing (Rabinovitch et al., 1990). Possibly because of its innate role in activating adaptive immune responses, it was not surprising that IL-1 receptor-deficient NOD mice had reduced development of diabetes (Thomas et al., 2004). Treatment with the IL-1 receptor antagonist, Anakinra, was shown to improve glucose control in patients with type 2 diabetes, which is not thought to be mediated by adaptive immune responses but has a significant inflammatory component (Donath and Mandrup-Poulsen, 2008). Interestingly, the drug mechanism appeared to involve a beneficial effect on β cells, reflected by an increase in the insulin:proinsulin ratio, rather than effect on reduced insulin sensitivity that had been thought to be the result of the inflammatory cytokine. β cells may be a source of IL-1, particularly in response to glucose, suggesting a destructive cycle in which hyperglycemia induces expression of the inflammatory mediator resulting in immune activation and further β cell destruction. Initial preclinical data do not suggest that IL-1 blockade alone will prevent or reverse type 1 diabetes, but this axis may be an important target of a combination strategy. Studies to evaluate the effects of IL-1 blockade in disease progression are in progress.

TNF-α and IFN-γ are directly cytotoxic to β cells, suggesting these cytokines as rational targets for immune therapy. However, TNF-α has a more complicated role in diabetes progression. Jacob et al. (1990) reported that TNF-α prevented development of insulitis and diabetes and even the adoptive transfer of diabetes by lymphocytes into young NOD mice. Moreover, neutralization of TNF-α accelerated diabetes in older mice but prevented disease at a younger age. These paradoxical effects may have led to reluctance for clinical translation, but a recent report by Mastrandrea et al. (2009) found that the soluble TNF receptor, Etanercept, reduced loss of C peptide responses in a small pilot trial.

Small-Molecule Protease Inhibitor Therapy

Small-molecule protease inhibitors are also under development for the treatment of T1D. The role of innate immune responses in T1D pathogenesis is further supported by the study by Koulmanda et al. (2008) in which infusions of alpha-1 antitrypsin (AAT), a serine protease inhibitor that protects tissues from enzymes produced from inflammatory cells, were found to reverse new-onset diabetes in NOD mice. Multiple effects were noted in the NOD studies including reduced insulitis, enhanced β cell regeneration, and improvement in peripheral insulin sensitivity. This nonconventional approach is now in clinical testing.

The small-molecule tyrosine kinase inhibitor, Gleevec, used widely for treatment of leukemia, was shown to prevent and reverse diabetes in NOD mice (Louvet et al., 2008). The effects appeared to be linked to inhibition of platelet-derived growth factor receptor (PDGFR) because targeting c-Abl kinase with sunitinib or c-Kit kinase and c-Fms kinase with another tyrosine kinase inhibitor showed marginal efficacy whereas soluble PDGFR reversed diabetes.

Aggressive Insulin Therapy

Lastly, aggressive insulin therapy has been tested for therapeutic efficacy in T1D. Shah et al. (1989) showed that use of a closed-loop system, in which patients with new-onset T1D were administered insulin to suppress endogenous insulin production, resulted in improved metabolic function, similar to more recent trials of immune modulators. It was possible that the intensive insulin therapy had an immune-modulatory effect, but this early observation also raises the question of whether reducing metabolic demand on the targets themselves might alter the immune response to islets.

Antigen-Specific Tolerance Approaches to T1D Therapy

The gold standard therapy for the treatment of autoimmune diseases, including T1D, would be the development treatment strategies in which only the pathogenic autoreactive T cells are inactivated safely and in an autoantigen-specific manner while leaving the remainder of immune system unperturbed, i.e., the induction antigen-specific immunologic tolerance. There are multiple strategies under development and/or currently being evaluated in T1D trials that are proposed to target multiple diabetogenic antigens and have been demonstrated to operative via a number of cell-intrinsic (anergy) and/or cell-extrinsic (Treg cells) mechanisms.

Insulin Therapy

Insulin therapy has been widely studied in both animal models of T1D as well as in human prevention and new-onset trials. In several autoimmune disease models, mucosal exposure to autoantigens induces tolerance largely via induction of a variety of Treg cells (Faria and Weiner, 2005). Insulin and proinsulin molecules have been identified to play a prime role in the initiation of the autoimmune process that ultimately leads to destruction of β cells and onset of clinical diabetes. Since the early 1990s, mucosal exposure of insulin and many of its immunogenic epitopes has been used for diabetes prevention in animal models. Oral insulin at a dose of 1 mg twice a week for 5 weeks followed by weekly treatment was able to delay diabetes onset and reduce diabetes incidence in NOD mice (Zhang et al., 1991). Adjuvants such as cholera toxin B subunit could significantly reduce the amount of antigen (insulin) needed to microgram amounts (Bergerot et al., 1997). Similarly to oral treatment, intranasal aerosol insulin treatment of prediabetic NOD mice also significantly delayed diabetes incidence in NOD mice (Aspord and Thivolet, 2002; Harrison et al., 1996). In addition to whole insulin, insulin-derived peptides, such as $B_{9\text{-}23}$, mutated proinsulin peptide B24-C33, and proinsulin II, have also been shown to be efficacious in prediabetic NOD mice (Chen et al., 2001; Daniel and Wegmann, 1996; Martinez et al., 2003).

Despite the persistence of even "clinically significant" levels of residual insulin and the potential for recovery of dysfunctional β cells with immune therapy at the time of diagnosis, prevention of T1D will have a greater impact than treatment approaches. Autoimmunity to islets can be identified ≥ three years before presentation with hyperglycemia in many individuals. Interventions that are effective at onset would be postulated to be effective in the prediabetic period. In addition, by intervening at an early stage, antigen-specific approaches might be more effective because the repertoire is more restricted and the number of different effectors that are involved is more restricted. Based on the success in animal models, clinical trials of oral or nasal insulin have been conducted in humans. These trials can be divided into prevention trials in prediabetics and therapeutic trials in recent-onset diabetics.

Human prevention trials have included a double-blinded crossover safety study conducted in 38 individuals with antibodies to one or more islet antigens which showed that intranasal insulin was safe in that it did not accelerate loss of β cell function in individuals at risk for type 1 diabetes but instead induced an increase in antibody and a decrease in T cell responses to insulin consistent with mucosal tolerance (Harrison et al., 2004). The subsequent DPT-1 tested the efficacy of oral insulin in 388 prediabetic patients who were first- and second-degree relatives of T1D patients and were also classified as at increased risk for developing T1D by genetic, immunological, and metabolic staging (Skyler et al., 2005; Sosenko et al., 2006). Oral insulin therapy did not delay or prevent type 1 diabetes. However, in subgroup analysis, it appeared that there might be a potential benefit in diabetes prevention in those subjects with higher autoantibody levels. A more recent prevention trial using intranasal insulin conducted in 224 Finnish children with genetic and immunological risks for developing T1D showed that nasal insulin administration at 1 unit/kg/day initiated soon after detection of autoantibodies had no beneficial effect on diabetes prevention (Näntö-Salonen et al., 2008). Furthermore, children positive for three of four autoantibodies before initiation of treatment appeared to be at possibly increased risk for accelerated onset of diabetes. This is a classic example of where preclinical studies were not predictive of the outcome of a human trial.

Several explanations have been offered for the failure of these trials, including insufficient dosing as well as the fact that by the time an individual is identified with autoantibodies, the disease process is well established. Therefore, the opportunity to intervene before the autoreactive repertoire is expanded via epitope spreading (Miller et al., 2007), i.e., before the appearance of multiple autoantibodies, using tolerance strategies with or without broader immunosuppressive agents, should be further explored. In addition, this may also reflect the complexity of mucosal immunology. Depending on pre-existing milieu, both tolerance and immunity are potential outcomes after mucosal antigen exposures. This could explain why possible disease acceleration has been observed with mucosal insulin therapy in certain subpopulations. Again, understanding individual immune responses elicited by mucosal insulin therapy based on the dose, route, frequency, duration, and stage of disease at which therapy is instituted will probably significantly enhance our ability to design individualized mucosal insulin therapy that will be safe and efficacious.

There have been a number of human new-onset trials using insulin therapy. Two published trials examined the effect of oral insulin therapy on residual β cell function in recent-onset T1D patients. In the immunotherapy diabetes (IMDIAB) trial, a total of 82 patients with clinical type 1 diabetes were randomized to receive oral insulin at 5 mg/day or placebo (Pozzilli et al., 2000). At a 1 year follow-up, there was no difference between the insulin-treated and the placebo-treated groups with respect to mean C-peptide secretion, requirement for insulin therapy, or IgG insulin antibodies. Furthermore, in patients younger than 15 years, a tendency for low C-peptide at 9 and 12 months was observed in the oral insulin group, suggesting an acceleration in the decline of β cell function. In the Diabete Insuline Orale group (ORALE) trial, 131 new-onset T1D patients were randomized to a low-dose (2.5 mg/day) or a high-dose (7.5 mg/day) oral insulin versus placebo for 1 year, and again no benefit was observed in preventing deterioration of β cell function (Chaillous et al., 2000). These results are consistent with those seen in murine models where oral insulin was shown not to reverse new-onset diabetes (Fousteri et al., 2007). Interestingly, if nasal insulin therapy is used in combination with anti-CD3 therapy, a significant benefit in reversing recent-onset diabetes is then achieved in two animal models of autoimmune diabetes (Bresson et al., 2006). Expansion of insulin-specific Treg cells producing IL-10, TGF-β, and IL-4, and possibly their modulation of antigen-presenting cells in local draining lymph nodes, were proposed as likely mechanisms. These findings should provide the basis for using combinatorial therapies in future trials for humans with recent-onset diabetes as discussed below.

Interestingly, a more recent phase I study using a single intramuscular injection of human insulin B chain in incomplete Freund's adjuvant in 12 subjects with recent-onset diabetes showed that this therapy led to the development of lasting (at a 2 year follow-up) insulin B chain-specific $CD4^+$ Treg cells (Orban et al., 2009). This study provides the basis for testing this

modality of insulin B chain therapy in a larger T1D trial. Another ongoing phase I–II clinical trial of subcutaneous BHT-3021, a plasmid encoding proinsulin, is testing the safety, dose, and preliminary efficacy of this therapeutic modality in recent-onset T1D patients (http://www.bayhilltherapeutics.com and http://jdrf.org).

Glutamate Decarboxylase 65

Immune therapies targeting glutamate decarboxylase 65 (GAD65), an early target of autoantibodies during the initiation of T1D (Kaufman et al., 1993; Tisch et al., 1993), have also been tested in both animal models and human T1D. Interestingly, the initial antigenic region is confined to a few epitopes near the C terminus of the GAD protein but later spreads intramolecularly to other GAD determinants, followed by further intermolecular spreading to other β cell antigens. Consequently, tolerance with intravenous or intrathymic injections of GAD in female NOD mice at 3 weeks of age eliminates the anti-GAD T cell responses, as well as subsequent spreading of the cascade of T cell responses to other β cell antigens and the development of insulitis or clinical diabetes (Tisch et al., 1993). Intravenous injections of GAD during the later stages of disease still effectively blocked disease progression in prediabetic mice and protected syngeneic islet graft survival in diabetic NOD mice (Tian et al., 1996). The identification of $CD4^+$ Treg cells in GAD-treated mice suggests a major role for bystander suppression in the induction of tolerance by treatment with this autoantigen, which raises the question of whether GAD is targeted early in T1D (Tisch et al., 1998).

Detection of GAD65 antibodies in the sera of prediabetic individuals is a reliable predictive marker for the progression to overt diabetes (Leslie et al., 1999). Promising preclinical data in the NOD model prompted two clinical trials using alum-formulated human recombinant GAD65. A phase II safety and dose-finding trial conducted in patients with latent autoimmune diabetes in adults (LADA) (Agardh et al., 2005) showed the drug to be safe, and administration of two 20 μg subcutaneous doses 1 month apart led to an increase of fasting and stimulated C-peptide at 24 weeks compared to baseline, a benefit that was associated with an increase in $CD4^+CD25^+$ Treg cells. The second trial used the 20 μg dosing regimen in recent-onset T1D children between 10 and 18 years of age (Ludvigsson et al., 2008). A slower decline of fasting and stimulated C-peptide was observed in the GAD-alum group compared to the placebo. More importantly, the protective effect of GAD-alum was preferentially exhibited in those who received treatment within 6 months of diagnosis, suggesting that the autoimmune process is more susceptible to GAD-based modulatory therapy if initiated at an earlier stage.

Heat Shock Protein

Therapies targeting *heat shock protein* (*hsp*) have also been tested in animal models and human trials. Early controversies existed as to whether heat shock proteins (hsp) were true autoantigens implicated in the pathogenesis of T1D (Atkinson et al., 1991). However, extensive preclinical studies using the hsp60 peptide p277 demonstrated efficacy of peptide vaccination in halting disease progression in the NOD mice (Elias and Cohen, 1995; Elias et al., 1991). p277 treatment appeared to promote Th2 cell type response with upregulation of IL-10 and IL-13 and downregulation of IFN-γ (Elias et al., 1997; Jin et al., 2008). p277 also exerts inhibitory effects on the innate immune system via signaling through TLR-2, leading to inhibition of inflammatory lymphocyte chemotaxis (Nussbaum et al., 2006).

The equivalent of human hsp60 p277 is a 24 amino acid synthetic peptide derived from the C terminus of the human hsp60, termed DiaPep277. Several phase I and II clinical trials in human T1D patients have been completed in Europe, and phase III trials are underway. A phase II trial was conducted in patients with established T1D but with residual β cell function (Huurman et al., 2007) and used a dose range of subcutaneously administered DiaPep277. Results showed a trend of dose-dependent preservation of stimulated C-peptide secretion. Three additional trials were performed in new-onset T1D patients (Lazar et al., 2007; Raz et al., 2001; Schloot et al., 2007). Two of these trials enrolled adult TID patients, whereas the third enrolled pediatric T1D patients. The adult trials showed significantly better preservation of insulin synthesis as measured by C-peptide production in the treated groups compared with placebo, but this effect was not seen in the pediatric trial. Similar results were observed in one other trial performed in pediatric patients (Schloot et al., 2007), although in children with less aggressive disease progression based on genetic background, there appeared to be a trend to better preserved C-peptide at the end of the study period. In summary, phase II trials with DiaPep277 have shown some promise in preserving residual β cell function, which appears to be less effective in patients with more aggressive disease. A phase III trial is underway with results expected in 2011.

Insulin-Coupled, ECDI-Fixed Antigen-Presenting Cells

An alternative technique for effective tolerance induction for treatment of autoimmune diseases is the administration of autoantigenic peptides covalently crosslinked to cellular vehicles via ethylene carbodiimide (ECDI; reviewed in Miller et al., [2007]). In preclinical models of various autoimmune diseases, this approach involves chemically crosslinking autoantigenic proteins or peptides to syngeneic splenic leukocytes via ECDI (Miller et al., 1979). It has been demonstrated that intravenous injection of these antigen-coupled splenocytes (Ag-SPs) is a highly efficacious method for the induction of tolerance for *both* the prevention and the treatment of a variety of immune-mediated disorders in animal models, including the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS) (Kennedy et al., 1990; Miller et al., 2007; Tan et al., 1992), T1D in the NOD mouse (Fife et al., 2006) (S. Prahad, A.P. Kohm, and S.D.M., unpublished data), and islet transplant rejection (Luo et al., 2008). Ag-SP tolerance induced by this method is indirect in that the input Ag-SPs, which are induced to undergo rapid apoptotic cell death after ECDI fixation (Turley and Miller, 2007), are uptaken in the host spleen, which is critical for tolerance induction because splenectomy abrogates tolerance induction to both autoantigens and alloantigens (D.R. Getts, A.M. Martin, X.L., and S.D.M., unpublished data). ECDI-fixed cells accumulate in the splenic marginal zone and induce splenic antigen presenting cells (APCs) to upregulate inhibitory costimulatory molecules (i.e., PD-L1) and to secrete regulatory cytokines (i.e., IL-10 and TGF-β), leading to unresponsiveness via two independent but synergistic mechanisms—T cell-intrinsic PD-L1-PD-1-mediated anergy and activation of iTreg cells as demonstrated for regulation of EAE (Miller et al., 2007), T1D (Fife et al., 2006, 2009), and allogeneic islet cell transplantation

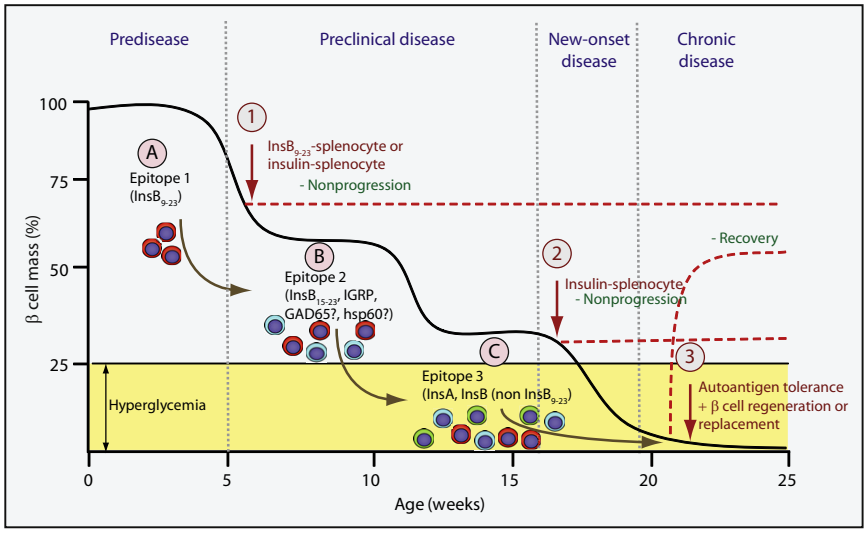

Figure 1. Model of Epitope Spreading and Tolerance Therapy in the Pathogenesis of Type 1 Diabetes in the NOD Mouse

Progression of T1D in the NOD mouse involves the sequential activation of autoreactive T cells to multiple diabetogenic epitopes via epitope spreading; these T cells accumulate until clinical diagnosis when sufficient autoreactive effector cells are present to cause destruction of the majority of the β cell mass. The insulin B chain epitope 9-23 ($InsB_{9-23}$) (A, red effector cells) appears to be the initiating or very early pathogenic diabetogenic epitope in the NOD mouse on the basis of the ability of tolerance induced by ECDI-fixed splenocytes coupled with either intact insulin or $InsB_{9-23}$ in 4- to 6-week-old mice to inhibit development of clinical diabetes (1). As β cell destruction continues, responses to additional islet antigens, e.g., $InsB_{15-23}$ and/or IRGP (B, blue effector cells) and eventually epitopes on the insulin A or B chains (C, green effector cells) are activated. Epitopes on the InsA or InsB chain (outside of B_{9-23}) epitopes appear to be dominant at the stage of transition to overt disease (loss of approximately 75% of islet mass) based on the ability of tolerance induced by insulin-coupled, but not $InsB_{9-23}$-coupled, splenocytes to ameliorate disease progression in 18–20-week-old NOD mice (2). Recovery from (i.e., reversal) chronic T1D when all of the β cells have been destroyed would be expected to require a combination of tolerance to the autoantigens that were responsible for initial β cell destruction and a β cell regeneration and/or replacement strategy that may require allo- or xenoantigen tolerance in therapies involving islet cell transplantation (3). A similar pattern of epitope spreading is postulated to occur in human T1D.

(Luo et al., 2008). Reprocessing and representation of antigens coupled to apoptotic Ag-SP debris by host splenic dendritic cells gives this strategy the advantage that tolerance to autoantigenic epitopes can be induced by cellular carriers fixed with intact proteins or even crude homogenates of the disease target organ (Kennedy et al., 1990). The mechanisms of Ag-APC tolerance are fundamentally different from tolerance strategies using mucosal antigen administration or alum injections in that unresponsiveness is exquisitely antigen specific and does not appear to involve bystander suppression (Vanderlugt et al., 2000). This tolerance induction method is currently being tested in a recently initiated magnetic resonance imaging (MRI)-controlled phase I–IIa clinical trial in new-onset relapsing-remitting MS patients at the Center for Multiple Sclerosis, University of Hamburg, Germany. The trial is examining the effects of tolerance induction by using peptide-coupled, ECDI-fixed autologous peripheral blood leukocytes (Ag-PBLs) coupled with a cocktail of seven myelin peptides (encompassing immunodominant MS-associated CD4 T cell epitopes on three separate myelin proteins) in an attempt to inhibit the potential of epitope spreading to multiple endogenous myelin epitopes. A second clinical trial using insulin-coupled PBLs for prevention of T1D is currently under development by the Immune Tolerance Network (http://www.immunetolerance.org). The ability to simultaneously target multiple myelin epitopes has been demonstrated in several mouse EAE models employing Ag-SP tolerance (Smith and Miller, 2006) and is likely to be important in T1D because epitope spreading is an important component of disease pathogenesis in the NOD mice (Figure 1). Disease appears to be initiated by T cell responses to the immunodominant $InsB_{9-23}$ epitope and then spread to other insulin epitopes as illustrated by the finding that tolerance induced in young NODs by splenocytes coupled with either intact insulin or $InsB_{9-23}$ inhibits development of T1D, but prevention of new-onset disease (18–20 weeks in our colony) can only be induced by tolerance to intact insulin (S. Prasad, A.P. Kohm, and S.D.M., unpublished data). This suggests that $InsB_{9-23}$ is an initiating diabetogenic epitope in NOD mice, as supported by a recently reported genetic approach (Nakayama et al., 2005), and that the response evolves to target other insulin epitopes outside of this region as mice transition to overt hyperglycemia. A similar scenario of epitope spreading is postulated to occur in human T1D and will influence the antigenic specificities needed to be targeted in tolerance-based immunoregulatory strategies.

Combination Therapies

The lack of permanent remission of T1D with any single agent suggests that combination therapies may be required for treating T1D. A combination of approaches may be needed for effective prevention of disease or reversal of new-onset T1D. Various broader-spectrum immunoregulatory or suppressive agents used in combination or together with antigen-specific tolerance strategies have been tested in animal models of T1D and in a limited number of clinical trials.

Because effector T cell responses are highly influenced by the cytokines in the environment, combination of an agent that can create a tolerogenic environment with a diabetogenic antigen would be predicted to better modulate antigen response. Synergy has been observed in reversal of diabetes in the NOD and lymphocytic chroriomeningitis virus (LCMV) models of the disease when insulin peptide was administered intranasally together with anti-CD3 mAb (Bresson et al., 2006). Insulin peptide-specific T cells isolated from these mice exhibited regulatory function and produced IL-10 and TGF-β in response to antigen. This synergy probably involved both the reduction of the ongoing response by the anti-CD3 mAb in combination with the induction of antigen-specific Treg cells because neither treatment alone was able to induce the antigen-specific regulatory cells. Other drug combinations have shown synergistic effects in the NOD T1D model, e.g., synergy between IL-1 blockade with anti-CD3 mAb therapy (K.C.H., unpublished

data). Interestingly, despite the complementary effects on effector cells while promoting expansion of Treg cells, rapamycin negated the effects of anti-CD3 mAb on diabetes in NOD mice without altering the frequency or phenotype of T cells. Even mice that had been rendered normoglycemic with anti-CD3 mAb had their tolerance broken by treatment with rapamycin.

Other studies have combined immunologic approaches with approaches aimed at restoring β cell function to achieve glycemic control. For example, the combination of a glucagon-like peptide 1 (GLP-1) receptor agonist (Exendin-4) was found to augment β cell function in diabetic mice treated with anti-CD3 mAb or ATG (Ogawa et al., 2004; Sherry et al., 2007). There was little evidence for immune effects, but the insulin content of pancreatic β cells was increased, possibly by enhancing recovery of degranulated β cells that can be identified in islets at the time of diagnosis.

There are few completed human trials with combinations of immune modulators, in part because of the regulatory issues involved with testing unapproved drugs. Published studies have been limited to agents that have previously been approved for use in other illnesses. A combination trial of IL-2 with rapamycin, supported by the Immune Tolerance Network, is underway. This approach is based on the complementary actions of the two agents to cause activation-induced cell death with sparing and perhaps expansion of Treg cells. An older study involved the combination of azathioprine and prednisone, which showed efficacy comparable to other agents such as CSA (Silverstein et al., 1988). The most notable combination has been the use of autologous nonmyeloablative hematopoietic stem cell transplantation in subjects with new-onset T1D. Subjects received pretreatment with cyclophosphamide and granulocyte-colony stimulating factor (G-CSF) to expand $CD34^+$ cells that were harvested and reinfused after treatment of subjects with ATG and cyclophosphamide. Unlike the experience in other immune modulation trials, 14 of 15 patients were rendered insulin free for an average of 16 months (Couri et al., 2009; Voltarelli et al., 2007). Toxicity was a clear problem; oligospermia was seen in 10 of 22 subjects, and one case of pneumonia was reported. Nonetheless, the extent and duration of insulin recovery was unequaled by other approaches.

Concluding Remarks

Antigen-induced and/or antigen-specific Treg cell-mediated tolerance-based strategies targeting only autoreactive T cells in the absence of long-term application of broad-based immunoregulatory or suppressive drugs or antibodies are the targeted immunotherapies for prevention or early reversal of T1D. Ideally, the tolerance therapy would specifically target β cell antigens involved in initiation of disease pathogenesis as well as identified endogenous islet autoantigens, which may be recruited to become targets of the ongoing autoimmune disease process via epitope spreading. Antigen- or Treg cell-induced tolerance therapies must also be durable, i.e., have the ability to regulate the autoimmune response permanently or at least for many years after induction, perhaps acting in part via the activity of renewable populations of autoantigen-specific Treg cells. Depending on the status of the autoimmune repertoire at the time therapy is initiated, tolerance induction may also have to be combined with or induced shortly after a tolerable immunoregulatory treatment (small-molecule- or antibody-based), which can function to reduce the autoantigen-specific T cell frequency to a level that can be effectively and durably suppressed. In addition, additional drugs may be required in combination to promote β cell regeneration. Regardless of the tolerance method employed for therapy, early intervention in T1D patients is critical to prevent ongoing islet destruction and to establish a microenvironment conducive to allow for the recovery of a normal β cell mass from endogenous progenitor cells. The chances for disease prevention will be improved by the identification of biomarkers identifying patients at risk as early in the disease process as possible.

Cellular adoptive-transfer-based approaches have shown significant promises in preclinical NOD models, both in prediabetic and postdiabetic stages. Specifically, both ex vivo expanded nTreg cells or induced $CD4^+CD25^+Foxp3^+$ Treg cells (iTreg cells) have been shown to control ongoing autoimmunity and either prevent progression to overt diabetes or protect syngeneic islet grafts and/or allow unperturbed β cell recovery, thereby inducing diabetes remission in NOD mice (Godebu et al., 2008; Luo et al., 2007; Tang et al., 2004; Weber et al., 2006). It is unclear whether antigen specificity is critically important in this approach because both nonspecifically expanded or induced Treg cells and islet antigen-specific Treg cells have shown efficacy in controlling the disease. Additionally, it also appears that Treg cells of one antigen specificity may be sufficient in controlling ongoing autoimmunity that is probably caused by autoaggressive T cells of multiple islet antigen specificities (Luo et al., 2007; Tarbell et al., 2004). Clearly delineating these characteristics of Treg cell adoptive-transfer therapy will have significant impact on the design of future clinical trials using this modality.

Another strategy for enhancing Treg cell numbers in vivo is by dendritic cell-based therapy. It has been shown that direct injection of either dendritic cells from pancreatic draining lymph nodes or β cell antigen-pulsed immature dendritic cells protects prediabetic NOD mice from developing overt diabetes, possibly through the in vivo induction of Treg cells (Clare-Salzler et al., 1992; Lo et al., 2006). However, direct ex vivo dendritic cell therapy carries the potential risk of their acquiring an activated phenotype upon adoptive transfer, leading to ultimate immunity rather than tolerance. An alternative approach for targeting dendritic cells for tolerance induction is by the in vivo delivery of cognate antigens to steady-state dendritic cells through the endocytic receptor DEC 205 (Bonifaz et al., 2002). It has been recently shown that delivery of β cell antigens in such a fashion leads to deletion of diabetogenic $CD8^+$ T cells in the context of ongoing autoimmunity (Mukhopadhaya et al., 2008). Ultimately, adoptive cell therapies that target both the CD4 and the CD8 compartments (Han et al., 2005; Santamaria, 2008) may provide synergy for protection against ongoing autoimmunity.

The question then becomes what is the ideal therapy to treat patients with long-standing T1D who have presumably destroyed all or the majority of their β cell mass, perhaps including renewable β cell progenitor cells? Again, tolerance-based therapies would be ideal in early onset, but intervention late in disease would still require that pancreatic autoantigen-specific processes be targeted prior to the transplant of stem cells capable of differentiation into insulin-producing β cells or

by transplantation of allogeneic (islets harvested from cadaver donors) or xenogeneic (e.g., porcine islet) islet cells. The critical requirement for autoantigen tolerance in advanced disease is amply illustrated by the fact that healthy islets from young NODs transplanted into long-term diabetic NOD recipients are vigorously rejected because of the residual autoimmune responses (Tian et al., 1996) and by anecdotal human data where pancreas transplants from identical twins are rejected (Sibley et al., 1985). Assuming that the immunosuppressive drugs required for the conditioning and/or maintenance of allo- or xenografts may not be compatible with induction or maintenance of autoantigen-specific tolerance, future therapies attempting reversal of overt diabetes in long-standing T1D patients secondary to islet transplantation will probably require tolerance to diabetogenic autoantigens combined with tolerance to the alloantigens or xenoantigens on the donor islets, an approach currently under testing in the NOD model using ECDI-fixed cells (Luo et al., 2008).

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